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Multixenobiotic Resistance in *Urechis caupo* Embryos: Protection From Environmental Toxins

BARBARA HOLLAND TOOMEY AND DAVID EPEL

Hopkins Marine Station, Stanford University, Pacific Grove, California 93950

Abstract. *Urechis caupo* is a marine worm that lives and reproduces in sediments containing a variety of potentially toxic environmental chemicals (xenobiotics). Its embryos have a multixenobiotic transporter, which is similar to the multidrug transporter in mammals, as indicated by their ability to transport a variety of moderately hydrophobic compounds such as dyes, drugs, and pesticides out of the cells. The cell membranes of the embryos contain a protein of approximately 145 kD that is immunologically related to the mammalian multidrug transport protein and that can be cross-linked by a photoactivatable substrate of the mammalian multidrug transport protein. The sediments in which the worm lives contain potential substrates for the transporter, indicating that this multixenobiotic transport activity may protect *Urechis* embryos from naturally occurring toxic compounds. Embryos of a sea urchin from a pristine environment do not have this transport activity and are sensitive to hydrophobic toxins. These data strongly support a role for multixenobiotic transport as a mechanism of protection from environmental toxins and indicate an unsuspected mode of protection in invertebrate embryos.

Introduction

Certain organisms live and reproduce successfully in areas rich with potentially toxic environmental chemicals, yet the way in which these organisms, and especially their embryonic stages, can grow and develop normally in these habitats is not well understood. For example, sediment-dwelling and filter-feeding organisms are indiscriminately exposed to a wide variety of hydrophobic compounds that may be cytotoxic. Here we report a mechanism that may allow the embryos of the echiuran worm *Urechis caupo* to survive and develop normally in an environment that is rich in hydrophobic compounds. This mechanism is similar to multidrug resistance (MDR) in mammalian cells and to multixenobiotic resistance (MXR) in several aquatic invertebrates. Multixenobiotic transport activity may indeed be one of several defense mechanisms that certain organisms have to help them resist natural toxins in their environment.

Multidrug resistance is a phenomenon in which cells become simultaneously resistant to several unrelated drugs, commonly through the increased expression of a membrane protein that reduces drug accumulation in the cells (reviewed in Gottesman and Pastan, 1988; Endicott and Ling, 1989; Gottesman *et al.*, 1991; Higgins, 1992). This protein, the multidrug transport protein or P-glycoprotein, is a member of a family of membrane proteins [ATP-binding cassette (ABC) transport proteins (Hyde *et al.*, 1990) or traffic ATPases (Ames and Lecar, 1992)] that are present in organisms from bacteria to humans and transport a variety of molecules using energy from ATP (McGrath and Varshavsky, 1989; Wu *et al.*, 1991; Samuelson *et al.*, 1990; Riordan *et al.*, 1989; Dudler and Hertig, 1992). The P-glycoprotein-mediated MDR confers resistance by binding drugs and transporting them out of the cells in an energy-dependent manner (Hamada and Tsuruo, 1988; Ambudkar *et al.*, 1992; Doige *et al.*, 1992; Doige and Sharom, 1992).

The substrate specificity of the mammalian multidrug transport protein has been characterized; a common feature among substrates of the transporter appears to be moderate hydrophobicity, with most substrates having both hydrophobic and positively charged domains (reviewed in Gottesman *et al.*, 1991). Although the role of the multidrug transport protein in normal tissues is not known, a variety of endogenous substrates of the transporter have been identified, including steroids (Wolf and Horwitz, 1992; Ueda *et al.*, 1992), organic chemicals (Charuk and Reithmeier, 1992; Ichikawa *et al.*, 1991),

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peptides (Sharma *et al.*, 1992), and ions (Gros *et al.*, 1992). The tissue distribution of the protein suggests that it plays a normal role in the transport of metabolites and also protects certain tissues from potentially toxic compounds (Thiebaut *et al.*, 1987; Tatsuta *et al.*, 1992).

Kurelec and his colleagues (reviewed in Kurelec, 1992) found that several aquatic invertebrates have an activity similar to multidrug transport in mammals, and suggested that this activity rids the organisms of toxic environmental compounds and confers multixenobiotic resistance on these organisms (Kurelec and Pivcevic, 1989, 1991, 1992; Kurelec *et al.*, 1992; Kurelec, 1992; Cornwall *et al.*, unpublished data). Several adult invertebrates, including the sponges *Tethya aurantium*, *Geodia cydonium*, and *Verongia aerophoba* and the mussels *Mytilus galloprovincialis* and *Anodonta cygnea*, have xenobiotic transport activity, as evidenced by the presence of a verapamil-sensitive drug export and verapamil-sensitive drug binding in several tissues (Kurelec and Pivcevic, 1989, 1991, 1992; Kurelec *et al.*, 1992; Cornwall *et al.*, unpublished data). This transport activity is also related to the presence of a protein similar to the mammalian multidrug transport protein in sponges and mussels (Kurelec *et al.*, 1992; Waldmann *et al.*, unpublished data). In relation to pollution, Minier *et al.* (1993) found that oysters and mussels from a polluted location have a higher frequency of expression of the multixenobiotic transport protein than do oysters and mussels from a site with little pollution.

Urechis caupo lives and spawns in a habitat rich with naturally occurring hydrophobic compounds. Here we describe a cellular mechanism, related to MDR in mammals, that may allow the embryos to develop normally in this seemingly noxious environment. The juvenile and adult worms may also need this mechanism to inhabit the sediment. MXR may be one of several mechanisms that invertebrates can use as a defense against environmental toxins, and may be especially important in developing embryos to prevent the toxins from entering the cells and interfering with normal development.

Materials and Methods

Adult specimens of *Urechis* were collected from a mudflat north of Moss Landing Harbor in Moss Landing, California, and maintained in glass tanks with several inches of mud and with running seawater. Fresh worms were collected every 2–3 months. *Urechis* gametes were collected and fertilized according to Gould (1967). *Strongylocentrotus purpuratus* adults were collected from the rocky intertidal shore at Point Arena, California, and the gametes were obtained and fertilized according to Leahy (1986).

Dye Transport Assays

To measure dye accumulation over time, embryos were incubated in filtered seawater (FSW) with 1 μM rhodamine B in the presence or absence of 22 μM verapamil (an inhibitor of mammalian multidrug transport activity) at 16°C. At the indicated times after suspension in rhodamine with or without verapamil, an aliquot of embryos was removed and washed twice by hand centrifugation and resuspension in FSW. The fluorescence of 10 embryos was then measured within 5 min of washing. Each embryo was viewed with a 40× water immersion lens on a Zeiss epifluorescence microscope fitted with a photosensor. An aperture was closed to a uniform diameter over each cell, and the light emitted by the cell was shunted to the photosensor. The light was then converted to a voltage that was quantified by a voltmeter and is expressed as relative fluorescence units. Error bars represent standard deviations. Unless otherwise indicated, each graph represents a single experiment.

Photographs of fluorescent dye accumulation in embryos were taken with AGFAPAN (ASA100) black and white film. Embryos were incubated in 1 μM rhodamine in the presence or absence of verapamil as above, and at 1 h they were washed free of the drugs. The two groups of embryos were mixed together immediately prior to photography.

To measure efflux of rhodamine over time, embryos were incubated in 1 μM rhodamine B for 1 h, after which they were washed three times with FSW and incubated in FSW or FSW + 22 μM verapamil. At various times after the dye was washed out, aliquots of each sample were washed once with FSW, and the fluorescence of the embryos was measured as above.

To assess the affinity of the transport activity for rhodamine, embryos were incubated in rhodamine with or without 22 μM verapamil for 1 h and washed. The fluorescence of 10 embryos was then measured as above. The concentrations of rhodamine B in FSW used were 0.01 μM, 0.1 μM, 1 μM, 5 μM, and 10 μM.

Radioactive drug accumulation

To measure drug accumulation, fertilized *Urechis* eggs were incubated at 16°C in FSW containing a mixture of unlabeled forskolin and [^{3}H]forskolin (DuPont-NEN) (0.5 μM forskolin + 1 μCi of [^{3}H]forskolin/ml of embryos; S.A. of [^{3}H]forskolin = 26.2 Ci/mmol) ± 22 μM verapamil or ± 10 μM rhodamine B. Two 0.5-ml aliquots of each sample were removed after 1 h, and the embryos were washed two times by hand centrifugation and resuspension in FSW. The pellet was dissolved in 0.5 ml of 0.5 N NaOH + 5% Triton X-100 and mixed with 5 ml of Ecolume (ICN Biomedicals, Inc.). The radioactivity of the solution was measured using a liquid scintillation counter (Beckman LS-8000). The values are the average of duplicate samples, with error bars indicating the standard deviations.

Toxicity assays

The effect of two cytotoxic drugs on cell division was measured in developing *Urechis* embryos in the presence and absence of verapamil. Fertilized eggs were incubated either in FSW ± 5 μM verapamil, FSW + 0.2 μM vinblastine ± 5 μM verapamil, or FSW + 0.5 μM vinblastine ± 5 μM verapamil. This experiment was also done with 2 μM or 4 μM emetine in place of the vinblastine. The embryos were allowed to develop at 16–18°C, and the number of embryos at the 1-, 2-, 4-, 8-, and 16-cell stages was counted 3 h after fertilization for each sample. The average number of cell divisions was determined by multiplying the percentage of embryos at each stage (minus the number of embryos that did not divide in the control samples) by the number of cell divisions that had occurred to produce that stage (*e.g.*, 4-cell stage = 2 divisions). Then the values for each cell division in each sample were added to obtain the number of cell divisions that occurred in that sample 3 h after fertilization. The average values of two experiments for vinblastine and three experiments for emetine are shown with error bars representing standard deviations.

Western blotting

For Western blot analysis, 200 μl of packed unfertilized eggs of *U. caupo* or *S. purpuratus* were homogenized in 200 μl of lysis buffer (Morris *et al.*, 1991). The proteins were then solubilized by adding 100 μl of 5% SDS and sonicating for several seconds. Tissues of the adult worm were dissected and chopped with a clean razor blade and treated in a similar manner as the eggs (a longer period of sonication and an additional 100 μl of SDS were used to solubilize the proteins in the adult tissues). Protein concentration was determined using the BCA protein assay (Pierce), and 40 μg of protein from each sample was then loaded onto the gel after the addition of an equal volume of mM Tris-Cl, pH 7.4, 10% sucrose, and 0.001% bromphenol blue. Proteins were separated on a 7.5% gel by SDS-PAGE, transferred to nitrocellulose (Schleicher & Schuell), and incubated first with the monoclonal antibody C219 (Centocor) and then with a goat anti-mouse antibody conjugated to alkaline phosphatase. The blots were developed using a substrate for alkaline phosphatase (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) (Mierendorf *et al.*, 1987).

Photolabeling and immunoprecipitation

Substrate binding to the *Urechis* multixenobiotic transport protein was determined by a competition assay using a photoactivatable substrate to label the protein and by testing to see whether other compounds could compete for that labeling. The photolabeling and immunoprecipitation experiments were essentially according to Morris *et al.* (1991); visualization of labeled proteins was by autoradiography. ^{125}I-6-AIPP-FSK (6-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]forskolin) (Morris *et al.*, 1991) was used to photolabel the membranes. For the competition experiment, egg membranes were incubated with 40 μM rhodamine B, 50 μM verapamil, 100 μM forskolin, 100 μM 1,9 dideoxyforskolin, or 2 μl of a chloroform:methanol extract of the sediment. For immunoprecipitation, the labeled membranes were dissolved by adding 20 μl of 50 mM Tris (pH 7.4), 20 μl of buffer A (2% SDS, 2 mg/ml BSA, 50 mM Tris, pH 7.4), and 200 μl of buffer B (1.25% Triton X-100, 190 mM NaCl, 50 mM Tris, pH 7.4). They were then immunoprecipitated with 10 μl of mdr(Ab-1) (Oncogene Science) and Protein A agarose beads (Pierce). After 2 h, the beads were washed four times with buffer C (0.1% Triton X-100, 0.03% SDS, 150 mM NaCl, 50 mM Tris, pH 7.4) and one time with buffer D (150 mM NaCl, 50 mM Tris, pH 7.4). Proteins were eluted with 35 μl of sample buffer containing 0.5 M Tris-HCl, pH 6.8, 20% glycerol, 10% (w/v) SDS, 0.1% bromphenol blue, and 4% β-mercaptoethanol. Samples were run on precast 10% Tricine SDS-PAGE gels (Novex) and stained with coomassie blue; the dried gels were exposed on x-ray film.

Sediment extraction

Sediment samples were taken from Moss Landing Harbor, California, at the same site where adult worms were collected. The sediment was frozen at −80°C within 1 h of collection and thawed just prior to use. To obtain pore water, approximately 40 ml of wet sediment was mixed well by shaking the sediment and its associated water vigorously. The sediment was removed by centrifugation, the pore water was extracted with an equal volume of chloroform:methanol (2:1), and the organic solvent phase was removed by evaporation. As a control, seawater that was not exposed to sediment was also extracted with organic solvent as above. The dried residue from each extraction (pore water or seawater) was dissolved into 100 μl of 95% ethanol prior to use in the dye assays. The effects of the extracts on dye transport were tested by incubating 5 ml of *Urechis* embryos in 100 nM rhodamine B in FSW ± 25 μl of the extract (2.4) or a dilution of the extract (1.2, 0.24). As a control, embryos were incubated in dye ± 22 μM verapamil. After 1 h, the fluorescence of the embryos in each sample was measured as above.

Results

Rhodamine accumulation and efflux

Multixenobiotic transport activity was measured using a fluorescence assay that follows the accumulation of rho-

damine dye, a substrate of the mammalian multidrug transporter (Neyfakh, 1988). In the absence of an inhibitor or competitive substrate of the transport activity (*e.g.*, verapamil), there is little rhodamine accumulation (little fluorescence increase), but when the transporter is inhibited, dye accumulates in the cells (fluorescence increase). This phenomenon is seen with *Urechis* embryos. Figure 1 shows the difference in intensity of dye in *Urechis* embryos at the 2-cell stage incubated in rhodamine ± verapamil for 1 h. Embryos in rhodamine + verapamil accumulate much more dye than embryos in rhodamine alone. To quantify this difference, measurements were made on single embryos over a 3-h period. In rhodamine alone the cells accumulate little dye. In the presence of rhodamine + verapamil, however, the fluorescence of *Urechis* embryos increases several-fold over the 3-h incubation. As shown in Figure 2A, uptake of dye in the presence of verapamil comes to an apparent equilibrium in about 1 h. Experiments with different batches of eggs showed slight variability in the fluorescence values, but the shape of the curves was the same.

In mammalian cells containing the multidrug transport protein, the accumulation of dye in the presence of verapamil is through the inhibition of this efflux pump (Fojo *et al.*, 1985), with verapamil acting as a competitive substrate or as an inhibitor. This is also true for *Urechis*. Embryos preloaded with rhodamine and washed into seawater show a rapid efflux of dye from the cells (Fig. 2B). However, in embryos washed into seawater + verapamil, this efflux of dye is retarded, as seen in the higher fluorescence of the embryos (Fig. 2B).

The *Urechis* transporter is effective at exporting low concentrations of substrate (1×10^{-8} to 5×10^{-6} *M*); there is increased dye accumulation in the presence of verapamil at concentrations ranging from 10 n*M* to 5 μM rhodamine B, but there is no difference in the fluorescence of embryos ± verapamil above 5 μM rhodamine (Fig. 3), suggesting substrate saturation above this concentration.

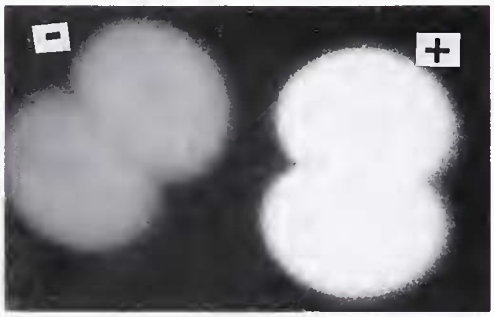

Figure 1. Photograph of *Urechis* embryos at the 2-cell stage incubated in 1 μM rhodamine ± 22 μM verapamil for 1 h: [(−) = rhodamine without verapamil and (+) = rhodamine with verapamil]. At the end of the 1-h incubation period, the embryos were quickly washed free of drugs, placed on a slide, and photographed.

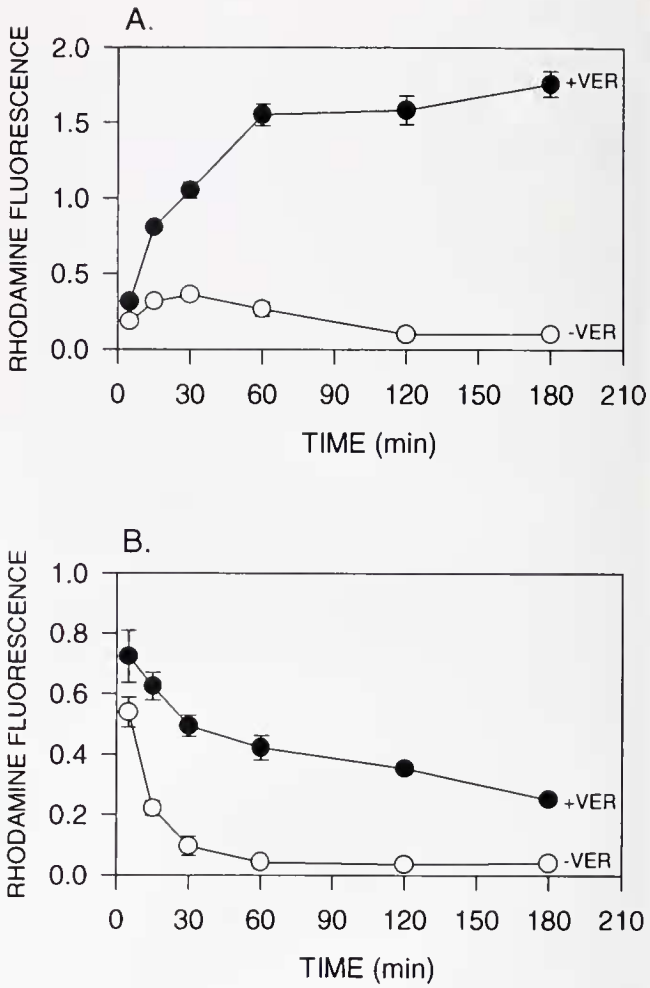

Figure 2. Accumulation and efflux of rhodamine B in *Urechis* embryos. (A) Rhodamine accumulation: Fertilized eggs were incubated in 1 μM rhodamine B (— ○ —) or in rhodamine plus 22 μM verapamil (— ● —), and the fluorescence was measured at various times. (B) Rhodamine efflux: Embryos were preloaded with 1 μM rhodamine for 1 h and washed into seawater (— ○ —) or seawater containing 22 μM verapamil (— ● —). Fluorescence of the cells in (B) was measured at various times after washing. Data points represent the average measurements of 10 eggs; error bars indicate standard deviations.

In addition to *U. caupo* embryos, we measured dye transport activity in embryos of the sea urchin *Strongylocentrotus purpuratus* to determine if the embryos of an organism collected from a relatively pristine environment also have this toxin defense mechanism. Figure 4 shows that, unlike *Urechis* embryos, the sea urchin embryos incubated in rhodamine have the same high fluorescence as embryos in rhodamine + verapamil.

We used competition for rhodamine export to indirectly identify substrates or inhibitors of the transport protein. Numerous drugs modulate the mammalian multidrug transporter (Hofsli and Nissen-Meyer, 1990; Ichikawa *et al.*, 1991), and many but not all of these inhibit rhodamine

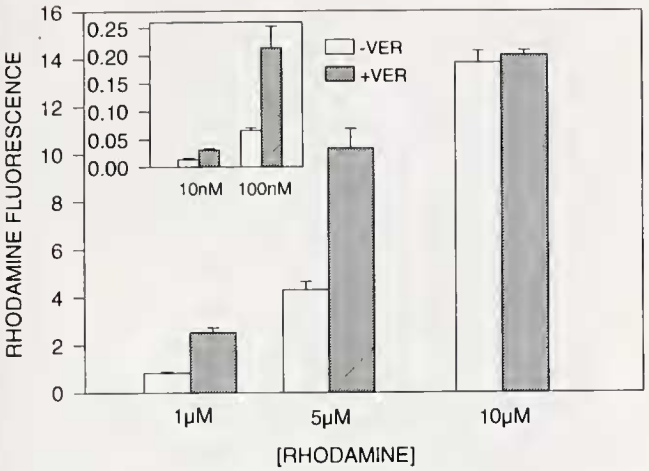

Figure 3. Relationsbip between rhodamine concentration and accumulation of dye ± verapamil. Embryos were incubated in increasing concentrations of rhodamine B ± 22 μM verapamil for 1 h. The inset shows fluorescence values of embryos in rhodamine concentrations of 10 nM and 100 nM

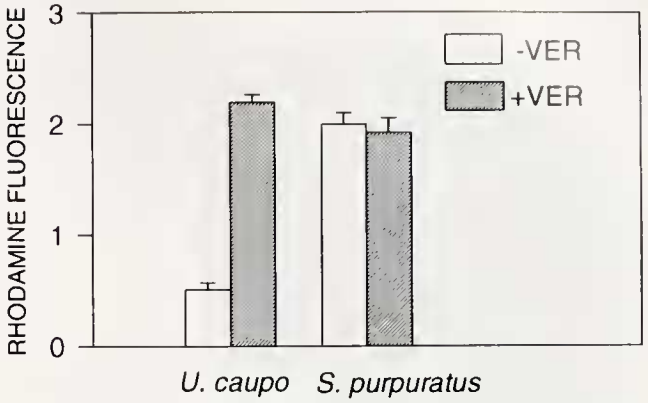

Figure 4. Dye transport activity in *Urechis caupo* embryos (*U. caupo*), and *Strongylocentrotus purpuratus* embryos (*S. purpuratus*). Fertilized eggs were incubated for 1 h in 1 μM rhodamine B ± 22 μM verapamil, washed with FSW, and the fluorescence of 10 cells was determined for each sample.

export from *Urechis* embryos. Potent inhibitors of rhodamine transport activity in embryos include verapamil, cyclosporin A, 1,9 dideoxyforskolin, forskolin, and quinidine (EC_{50}'s < 10 μM). Emetine, promethazine (EC_{50}'s ~ 20 μM) and vinblastine (EC_{50} ~ 40 μM) are less effective inhibitors, and colchicine does not affect dye transport in the embryos. (Data not shown.)

Radioactive drug accumulation

Multixenobiotic transport activity was also identified by assessing the accumulation of a radioactive substrate. In agreement with the fluorescence transport assay, more [^{3}H]forskolin accumulates in *Urechis* embryos in the presence of verapamil than in its absence (Fig. 5A). Similarly, rhodamine B inhibits the transport of [^{3}H]forskolin from the embryos (Fig. 5B).

Toxicity of drugs to developing embryos

Cell division in *Urechis* embryos incubated in vinblastine (Fig. 6A) or emetine (Fig. 6B) (substrates of the multixenobiotic transporter that inhibit cell division) is not greatly affected by low concentrations of these drugs. However, if multixenobiotic transport activity is inhibited with verapamil, the drugs now inhibit cell division as seen in Figures 6A and 6B. Verapamil had no apparent effect on the embryos during the first few cell divisions, but it caused a four- to fivefold decrease in the number of cell divisions of embryos when in combination with 0.2 μM vinblastine and a 350-fold decrease in the cell divisions in combination with 0.5 μM vinblastine. Embryos in 2 μM emetine + verapamil had two- to threefold decrease in the number of cell divisions, and embryos in 4 μM emetine + verapamil had a fourfold decrease in the cell divisions compared with embryos in emetine alone.

Western blotting

A protein related to the mammalian multidrug transporter was detected in *Urechis* egg membranes as assessed by Western blot, photoaffinity labeling, and immunoprecipitation experiments (Figs. 7 and 8). A monoclonal antibody to the conserved ATP-binding region of the mammalian multidrug transport protein (C219) identifies a protein of 140 kD in *Urechis* egg membranes and whole eggs (Fig. 7A) which is not abundant in the cytoplasmic fraction of the eggs. This protein is also identified by two

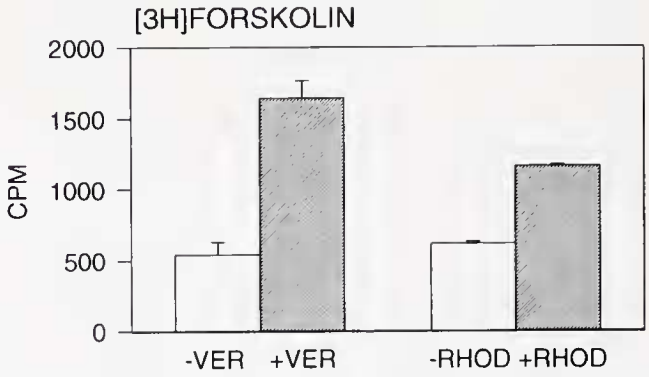

Figure 5. [^{3}H]Forskolin accumulation in *Urechis* embryos. On the left, embryos were incubated in 0.5 μM [^{3}H]Forskolin ± 22 μM verapamil for 1 h (−VER, +VER). On the right, embryos were incubated in 0.5 μM [^{3}H]Forskolin ± 10 μM rhodamine B for 1 h (−RHOD, +RHOD). The amount of radioactive forskolin in the cells was measured with a scintillation counter.

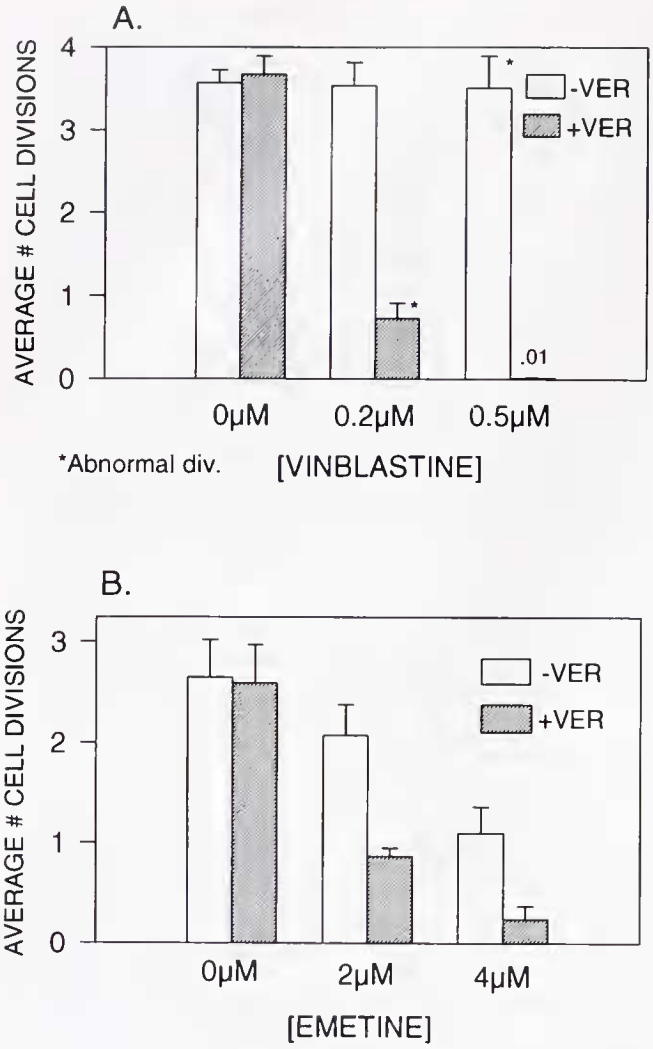

Figure 6. Effects of cytotoxic drugs on cell division. (A) Average number of cell divisions of embryos developing in FSW (0 μM), 5 μM verapamil (0 μM, hatched bar), and vinblastine at 0.2 and 0.5 μM ± 5 μM verapamil (+verapamil = hatched bars) after 3 h. Error bars indicate standard deviations from two experiments; * indicates that the embryos did not form normal cells upon division; .01 = the number of divisions that the embryos in 0.5 μM vinblastine + verapamil underwent. (B) Average cell divisions of developing *Urechis* embryos in FSW (0 μM), 5 μM verapamil (0 μM, hatched bar), or 2 and 4 μM emetine ± 5 μM verapamil (+verapamil = hatched bars) after 3 h. Error bars indicate standard deviations from three experiments.

polyclonal antibodies to the mammalian multidrug transport protein [mdr(Ab-1) and 4007] (data not shown). A major band appears at 140–145 kD in all the egg samples and in the mammalian MDR cells, but there are also some minor bands that could be proteolytic fragments (smaller bands) (Schinkel *et al.*, 1991) or multiples (larger bands) of the transport protein. The C219 antibody does not label a protein in *S. purpuratus* eggs (Fig. 7A). In a control experiment in which the C219 antibody was omitted, no bands were labeled in any of the samples (data not shown).

Western blot analysis of adult *Urechis* tissues shows that a major protein in both epidermal tissue and tissue from the anterior portion of the digestive tract stains heavily with an antibody to the mammalian multidrug transporter, but the band is lower in relative molecular mass (110 kD) than the egg or mammalian proteins (Fig. 7B).

Photolabeling and immunoprecipitation

A photoactivatable ^{125}I-forskolin derivative labels several proteins in mammalian cells including adenylyl cyclase and the multidrug transporter (Morris *et al.*, 1991). Proteins in *Urechis* egg membranes are also labeled by this forskolin derivative, and a major labeled protein of 145 kD in *Urechis* eggs is immunoprecipitated by mdr(Ab-1) (Fig. 8A), supporting the hypothesis that this protein is related to the mammalian multidrug transport protein. The labeling of a 140-kD protein by the forskolin derivative is inhibited by unlabeled forskolin, 1,9 dideoxyforskolin, rhodamine, and verapamil (Arrow, Fig. 8B).

MXR substrates in the sediment

Using a competition assay similar to the inhibition of dye export by verapamil, we tested the ability of compounds from the sediments in which the worm lives to inhibit dye transport (Fig. 9). Moderately hydrophobic compounds from the interstitial seawater associated with the sediments (pore water) inhibit dye transport in *Urechis* embryos (Fig. 9). These extracted compounds are water-soluble, and thus may be available to the embryos in the natural environment, but they are not found in seawater that is not associated with sediment (Fig. 9, SWEXT.). Furthermore, these environmental chemicals affect dye transport in the embryos at concentrations similar to those in the original pore water (Fig. 9, 1.2 = 1.2-fold concentration of the pore water). Undiluted pore water without chloroform:methanol extraction contains substrates of the transporter, although the results are variable (data not shown). Finally, the compounds extracted from the sediments inhibit the binding of the ^{125}I-forskolin derivative to the *Urechis* multixenobiotic transport protein (Fig. 8B; Sed. Extr.).

Discussion

This work demonstrates the presence of multixenobiotic transport activity in the embryos of a sediment-dwelling worm and suggests that this activity is responsible for protecting the embryos from toxic environmental compounds. In support of this role, we have shown that compounds from the sediments and some anthropogenic

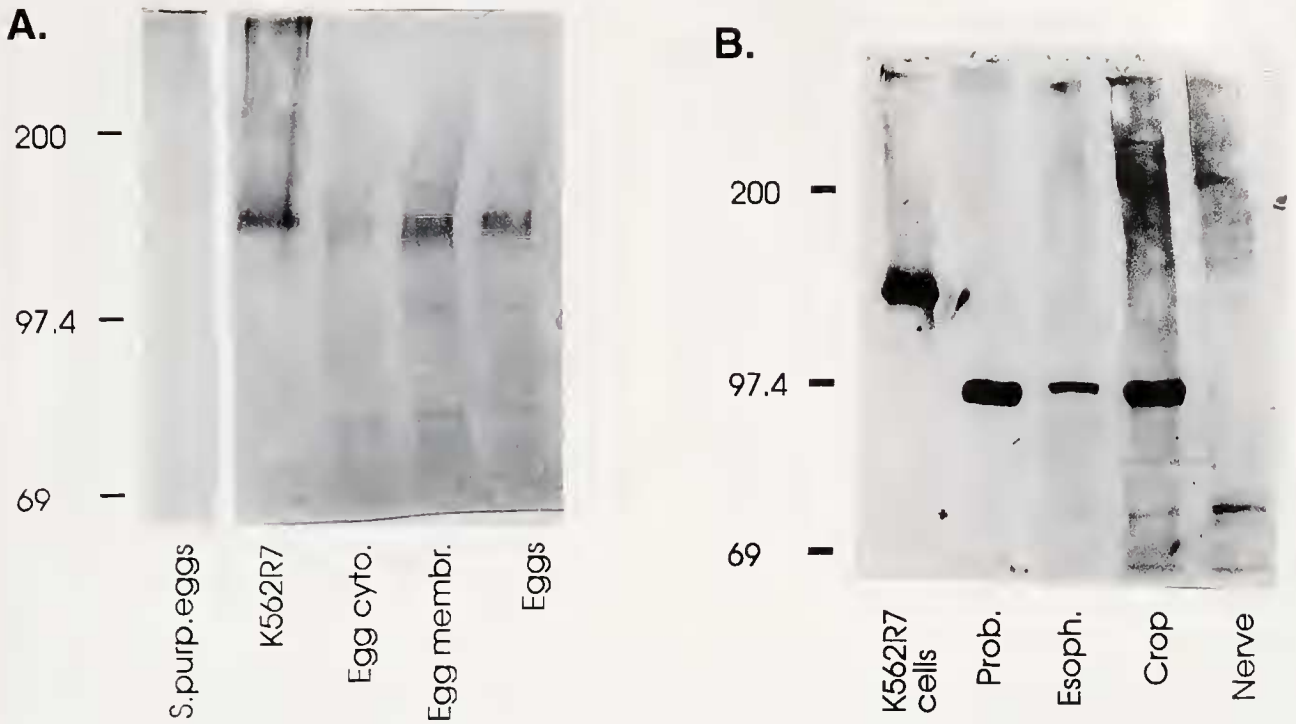

Figure 7. Western blot of the multixenobiotic transport protein in *Urechis* eggs and adult tissues. (A) Egg protein preparations were labeled with a monoclonal antibody to the mammalian multidrug transport protein (C219). *Urechis caupo* whole eggs (U.c. Eggs; lane 5), egg membranes (U.c. Egg membr.; lane 4) and egg cytoplasm (U.c. Egg cyto; lane 3). Mammalian K562R7 cells are an MDR+ control (K562R7 cells; lane 2). Eggs of *S. purpuratus* (S. purp. Eggs; lane 1) are a negative control. (B) Several tissues of the adult worm, including the proboscis, were labeled with C219 (Prob.; lane 2), esophagus (Esoph.; lane 3), crop (Crop; lane 4), nerve tissue (Nerve; lane 5), and K562R7 cells were again used as a positive control (K562R7 cells; lane 1).

compounds inhibit the labeling by forskolin of the multixenobiotic transport protein *in vitro* and inhibit dye efflux from *Urechis* embryos at low concentrations *in vivo*, indicating that these compounds may be substrates of a multixenobiotic transporter. Furthermore, two substrates of the transporter are more toxic to the embryos when the transport mechanism is inhibited by verapamil, suggesting a protective function.

Multixenobiotic transport activity in *Urechis* embryos is characterized by a verapamil-sensitive export of dyes and drugs similar to that found in MDR mammalian cells (Neyfakh, 1988; Hofsli and Nissen-Meyer; 1989). Using an indirect assay that measures the accumulation of dye in the cells in the presence or absence of potential substrates, our data show that the embryos have the capacity to export a variety of moderately hydrophobic compounds. The relative ability of each compound to inhibit dye transport in the embryos may relate to its affinity for the transporter relative to the dye or to its ability to enter the cells and interact with the protein. Results similar to those using the dye assay are also obtained when transport is measured with radioactive forskolin (Fig. 5), thus confirming and validating the results of the dye assay.

Another type of indirect but functionally relevant transport assay was done by measuring the effects of cytotoxic substrates on the embryos. When the multixenobiotic transporter is inhibited by verapamil, the cytotoxic substrates are more effective than when the embryos develop in the substrate alone (Fig. 6). Thus, when the multixenobiotic transport mechanism cannot function, toxic substrates have a greater effect on the embryos' development.

Characterization of the membrane proteins in *Urechis* reveals a protein at 140-145 kD that is similar to the mammalian multidrug transport protein. The fact that the protein is similar in size and is labeled by three different antibodies made to the mammalian multidrug transport protein indicates that *Urechis* embryos have a protein related by sequence, structure, or both to the mammalian protein. The adult worms also have a related protein, suggesting that the MDR-like protein is present in both the embryos and adults. The two proteins may be different but related, or the size difference between the egg and adult proteins may be due to proteolysis of the adult protein or differences in protein modifications. This question will be further studied using molecular techniques.

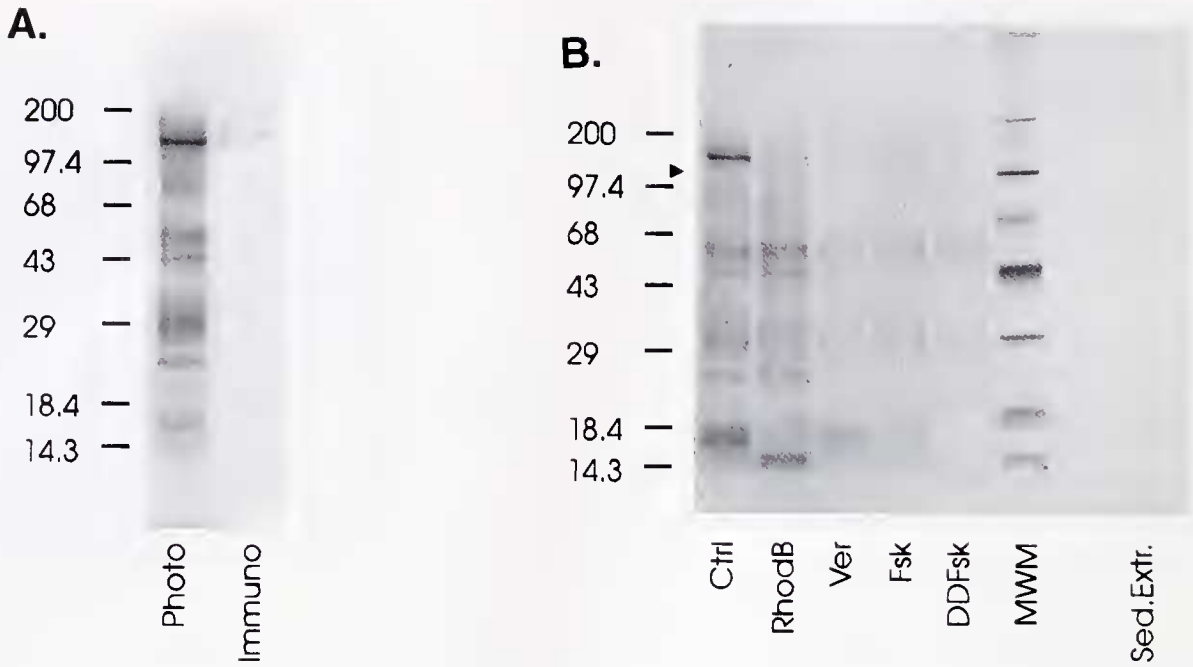

Figure 8. Photoaffinity labeling and immunoprecipitation: (A) *Urechis* egg membrane proteins labeled with a radioactive photoactivatable derivative of forskolin (Photo), and immunoprecipitation of the labeled proteins by an antibody to the mammalian multidrug transport protein [mdr(Ab-1)] (Immuno). (B) Egg membrane proteins were labeled with the forskolin derivative in the absence (arrow, Ctrl) and presence of an excess of unlabeled drugs: 40 μM rhodamine B (RhodB), 50 μM verapamil (Ver), 100 μM forskolin (Fsk), 100 μM 1,9 dideoxyforskolin (DDFsk), and sediment extract (Sed. Extr.).

The multixenobiotic transport protein in the eggs is also a drug-binding protein, as seen in the photolabeling experiments, showing that substrates of multixenobiotic transport activity interact directly with the 140- to 145-kD protein. This work suggests that the multixenobiotic transport protein, which is immunologically related to the mammalian multidrug transport protein, is also responsible for xenobiotic transport activity in *Urechis* embryos (*i.e.*, is responsible for the MXR phenomenon); a forskolin derivative specifically labels the *Urechis* multixenobiotic transport protein, and forskolin is a substrate for multixenobiotic transport activity.

Several of the drugs that competed for labeling of the 140-kD protein also decreased the labeling of some lower molecular weight proteins, possibly reflecting the nonspecific nature of these drugs. In particular, a protein at 20–25 kD was labeled much less in the presence of excess rhodamine B, forskolin, or 1,9 dideoxyforskolin. A protein of approximately 50 kD was also labeled; this could be adenylyl cyclase, a known target of forskolin.

A possible role of the mammalian multidrug transport protein in normal tissues is the transport of toxic dietary compounds or endogenous metabolites (Thiebaut *et al.*, 1987; Charuk and Reithmeier, 1992; Wolf and Horwitz, 1992; Roninson, 1992), and it has been hypothesized that a similar multixenobiotic transport protein may protect aquatic organisms from environmental toxins (Kurelec, 1992). *U. caupo* lives and spawns in mud flats rich with natural hydrophobic compounds, many of which adsorb to sediment particles (Yen and Tang, 1977). Although *Urechis* has planktonic larvae, they do not start swimming until several hours after fertilization and presumably proceed through the early developmental stages in close association with the mud. We have shown that the sediment in which the worm lives contains compounds that are potential substrates of the transporter (Fig. 9) and that interact with the *Urechis* multixenobiotic transport protein (compete for photolabeling, Fig. 8B). Exposure of the gel in Figure 8B on a phosphoranalyzer showed several faint bands in the Sed. extr. lane, but the band at 140 kD was not labeled (data not shown), indicating that compounds in the sediment specifically inhibited photolabeling of the multixenobiotic transporter. In further support of a role for the transporter in protection from environmental toxins (*i.e.*, in conferring MXR), we have preliminary evidence indicating that *U. caupo* embryos develop normally in sediment-exposed seawater (pore water), but that embryos of *Strongylocentrotus purpuratus*, which do not have transport activity, die before they reach the gastrula stage in this pore water (data not shown).

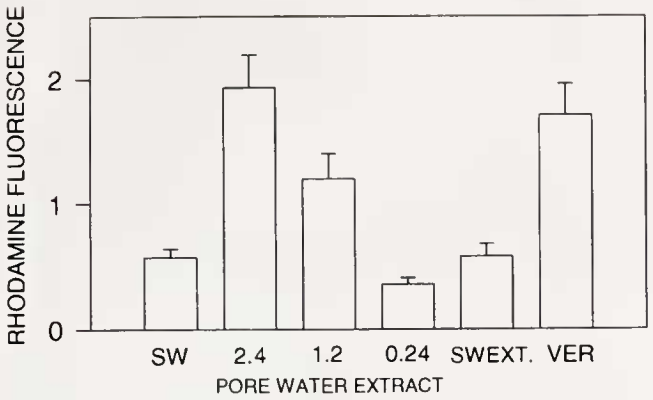

Figure 9. Competition for rhodamine export by pore water extract. Compounds leached from the sediments into the pore water and extracted into chloroform:methanol were used in the dye transport assay. Embryos were exposed to FSW containing 100 n*M* rhodamine B ± the following compounds for 1 h before the fluorescence was measured: SW = filtered seawater; 2.4 = pore water extracted into chloroform:methanol (the organic extraction of the pore water led to a 2.4-fold concentration of compounds from the original pore water to the dye assay); 1.2 = dilution of pore water extract to a 1.2-fold concentration of pore water compounds; 0.24 = dilution of pore water extract so that the compounds are less concentrated than in the original pore water; SWEXT = seawater extracted into chloroform:methanol; VER = 22 μM verapamil.

Many sediments are polluted with anthropogenic toxins as well as with natural hydrophobic compounds. Four moderately hydrophobic pollutants (CDEC, DCPA, pentachlorophenol, and 2-acetylaminofluorene) inhibit dye transport in *Urechis* embryos; the inhibition is similar to that produced by verapamil (Toomey and Epel, unpublished data). However, several pollutants of greater hydrophobicity (DDT, DDD, DDE, polychlorinated biphenyls, and benzo(a)pyrene) do not affect dye transport and presumably are not substrates of the transporter (Toomey and Epel, unpublished data).

All the compounds that act as substrates appear to be moderately hydrophobic, whether they are natural product drugs or manmade pollutants. Compounds of greater hydrophobicity may not be transported out of the cells; this may be because they do not enter the cells at all; because they become sequestered in the cell membrane; or because they do not interact with the transport protein, which may require substrates containing both hydrophobic and hydrophilic regions. Although these results indicate that the MXR mechanism will protect embryos from environmental toxins, this mechanism will not function if the substrates saturate the transport system. Thus, naturally occurring MXR in *Urechis* embryos and other marine organisms (Kurelec, 1992) is effective against low levels of moderately hydrophobic environmental toxins but may be ineffective against high levels of natural or anthropogenic pollutants.

Protection from a variety of toxic compounds is important for all life stages of an organism that lives and reproduces in an environment enriched with organic pollutants from natural or anthropogenic sources. Eggs and embryos are often the most sensitive stages of an organism's life, and the presence of multixenobiotic transport activity and other detoxification mechanisms may allow these embryos to survive, thus conferring multixenobiotic resistance. These protective mechanisms in both embryos and adults may account in part for the distribution of organisms and their ability or inability to live under exposure to environmental toxins. MXR could be an important first defense against moderately hydrophobic toxins that are attached to particulate matter in the organism's habitat or food supply (*e.g.*, for sediment-dwellers or filter-feeders). Organisms that do not have MXR may be unable to live in a habitat with an abundance of hydrophobic compounds.

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